DAMD17-86-C-6063

STRUCTURE-FUNCTION RELATIONSHIP OF HYDROPHILIIDAE POSTSYNAPTIC NEUROTOXINS

ANNUAL REPORT

ANTHONY T. TU
ROGER A. MILLER
NOBUHIRO MORI

MARCH 5, 1987

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6063

Colorado State University
Department of Biochemistry
Fort Collins, Colorado 80523

AD-A185 198

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS:
2a. SECURITY CLASSIFICATION AUTHORITY:	3. DISTRIBUTION/AVAILABILITY OF REPORT: See Distribution Statement at end of report.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE:	
4. PERFORMING ORGANIZATION REPORT NUMBER(S):	5. MONITORING ORGANIZATION REPORT NUMBER(S):
6a. NAME OF PERFORMING ORGANIZATION: Colorado State University; 6b. OFFICE SYMBOL (If applicable):	7a. NAME OF MONITORING ORGANIZATION:
6c. ADDRESS (City, State, and ZIP Code): Department of Biochemistry, Fort Collins, Colorado 80523	7b. ADDRESS (City, State, and ZIP Code):
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U. S. Army Medical Research & Develop. Command; 8b. OFFICE SYMBOL (If applicable):	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-86-C-6063
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 61102A; PROJECT NO. 3M161102BS12; TASK NO. AA; WORK UNIT ACCESSION NO. 098

11. TITLE (Include Security Classification)
Structure-Function Relationship of Hydrophiliidae Postsynaptic Neurotoxins (U)

12. PERSONAL AUTHOR(S)
Tu, Anthony T., Miller, Roger A., and Mori, Nobuhiro

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 3/1/86 TO 2/28/87	1987 March 5	

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Keywords - sea snake toxins, postsynaptic neurotoxins, chemical modification of toxins, hydrophilicity index of toxin and acetylcholine receptor, amino acid sequence of neurotoxins.

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

This annual report is comprised of three parts:

A. The hydrophilicity index of Lapemis toxin from Lapemis hardwickii sea snake and the α-subunit of the acetylcholine receptor of Torpedo californica have been determined and plotted versus the sequence position.

B. Lapemis toxin is a competitive inhibitor of α-bungarotoxin (αBTX) in binding to ACR. Two artinine residues out of three which are present in Lapemis toxin were modified by phenylglyoxal.

C. The major neurotoxin was isolated from the sea snake venom of Acalyptophis peronii captured in the Gulf of Thailand. Partial N-terminal sequence of the toxin was identified up to 37 residues. Ten more residues beyond residue No. 37 were also identified, but the exact positions of these ten residues have not yet been identified.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mrs. Virginia M. Miller	22b. TELEPHONE (Include Area Code): 301/663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

d. Title Page

Structure-Function Relationship of Hydrophiidae Postsynaptic Neurotoxins

The report is comprised of three parts. They are:

A. Hydrophilicity Analysis of Lapemis Toxin and Acetylcholine Receptor α-Subunit

B. Isolation and Chemical Modification of Lapemis Toxin from Lapemis hardwickii Toxin

C. Isolation and Amino Acid Sequence of Neurotoxin from the Venom of Sea Snake Acalytophis peronii

e. Summary

A. The hydrophilicity index of Lapemis toxin from Lapemis hardwickii sea snake and the α-subunit of the acetylcholine receptor of Torpedo californica have been determined and plotted versus the sequence position. The results show that the sea snake neurotoxin is quite hydrophilic and suggests an interaction at a hydrophilic region of the acetylcholine receptor. The analysis results of the acetylcholine receptor subunit support the known information about the intramembrane subunit. Other studies' information has been considered along with the hydrophilicity results and a model of binding interaction is proposed.

B. Lapemis toxin, the major toxin, was isolated from the venom of Lapemis hardwickii captured in the Gulf of Thailand. Acetylcholine receptor (ACR) was isolated from the electric organ tissue of Torpedo californica. Lapemis toxin is a competitive inhibitor of α-bungarotoxin (αBTX) in binding to ACR. The arginine residues out of three which are present in Lapemis toxin were modified by phenylglyoxal. The modification experiment will be done on the ACR bound neurotoxin in the future.

C. The major neurotoxin was isolated from the sea snake venom of Acalyptophis peronii captured in the Gulf of Thailand. The molecular weight was approximately 6,500. Acalyptophis toxin bound to acetylcholine receptor and was a competitive inhibitor of α-bungarotoxin binding. Partial N-terminal sequence of the toxin was identified up to 37 residues. Ten more residues beyond residue No. 37 were also identified, but the exact positions of these ten residues have not yet been identified. The complete sequence will be identified in the near future.

f. Foreword

A. Hydrophilicity Analysis of Lapemis Toxin and Acetylcholine Receptor α-Subunit

In order to elucidate the theoretical explanation of the neurotoxin (NT) acetylcholine receptor (ACR) interaction, the hydrophilicity index analysis was employed.

Lapemis toxin is a short-chain postsynaptic neurotoxin found in the venom of the sea snake Lapemis hardwickii. Lapemis toxin is a single polypeptide of 60 amino acids. The isolation, characterization, and sequence for Lapemis toxin have been determined by Fox et al., 1977.

The acetylcholine receptor (ACR) is a ligand-gated ion-channel transmembrane glycoprotein which, in response to the binding of acetylcholine, mediates the translocation of cations across the plasma membrane in which the ACR resides.

The ACR has an overall molecular weight of approximately 270,000 and is comprised of four non-identical subunits with molecular weights of 40,000 (α), 50,000 (β), 60,000 (γ), and 65,000 (δ), and the stoichiometry of $\alpha_2\beta\gamma\delta$. The α-subunit of the ACR has 437 amino acid residues and one site of glycosylation of aspargine 141 (Martinez-Carrion et al., 1975; Raftery et al., 1980; Noda et al., 1982; Noda et al., 1983; Claudio et al., 1983; Nomoto et al., 1986).

Sea snake neurotoxins bind to the α-subunit of the ACR and inhibit the binding of acetylcholine and inhibit the conformation change in the ACR thus blocking the depolarization of the myocytes that occurs in normal nerve transmission to muscles (Endo et al., 1986).

It is well known that one of the most important parameters of protein-protein interaction and protein folding and structure is due to hydrophobic-hydrophilic interactions. To better understand the ACR-NT interaction, the hydrophilicity index analysis has been done. Through this analysis the nature of the interaction has helped narrow the regions of importance between these two proteins. The more that is learned about this interaction will eventually lead to the exact chemical nature of the ACR-NT binding. With this knowledge, the structure-function of the normal functioning ACR and pathological conditions involving the ACR will be better understood.

B. Isolation and Chemical Modification of Lapemis Toxin from Lapemis hardwickii Toxin

Sea snake neurotoxins inhibit nerve transmission at the neuromuscular junction. Lapemis toxin, the major toxin isolated from Lapemis hardwickii venom, strongly binds to acetylcholine receptors. The toxin is one of many snake neurotoxins extensively investigated. It consists of 60 amino acid residues with molecular weight of 6,800. Its amino acid sequence was also established. However, the exact mechanism of binding to acetylcholine receptor has not been elucidated yet. In order to understand the interaction of neurotoxin to acetylcholine receptor, neurotoxin-ACR complex will be modified. It is assumed that the residues involved in the ACR binding should not be modified. As control, arginine residues of free neurotoxin was modified first and the result will be compared with that of bound toxin.

C. Isolation and Amino Acid Sequence of Neurotoxin from the Venom of Sea Snake Acalytophis peronii

Sea snakes (Family: Hydrophiidae) are abundant in the tropical and subtropical zones of the Indian Ocean and the Pacific Ocean. On the coast of the Gulf of Thailand there are numerous numbers and varieties of sea snakes. Lapemis hardwickii's venom was extensively studied. Acalyptophis peronii was also a quite common sea snake in the Gulf of Thailand, but its venom has not been investigated yet. Therefore, we don't know anything about the nature and structure of neurotoxins present in A. peronii venom. Three grams of venom were obtained in 1979. The amount of the venom is large enough to isolate the major neurotoxin; therefore, the isolation and amino acid sequence determination were made.

In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

g. Table of Contents

i. Body of the Report

A. Hydrophilicity Analysis of Lapemis Toxin and Acetylcholine Receptor α-Subunit by Anthony T. Tu, Roger A. Miller, and Nobuhiro Mori

Methods

Since the sequences of Lapemis toxin and the α-subunit of the acetylcholine receptor are known, the application of the hydrophilicity analysis was possible.

A computer program was developed for the Apple IIe personal computer based on the program by Hopp and Woods, 1983. The program takes the given sequence and assigns the hydrophilicity value to each amino acid. These values are assigned based on solvent parameter work by Levitt, 1976. The values of the hydrophobic solvent parameters s are taken as the measured free energy of transfer from water to ethanol in kcal/mol. The values assigned to each of the twenty amino acids are shown in Table 1. The values range from +3 to -3.4 with hydrophilic residues indicated by positive values and hydrophobic residues indicated by negative values. The program then calculates the average hydrophilicity value of a moving hexapeptide window through given sequence, and this average value is plotted versus the first amino acid residue of each window.

The sequence information for Lapemis hardwickii Lapemis toxin (NT) was taken from Fox et al., 1977.

The sequence information for the α-subunit of the Torpedo californica ACR was taken from Noda et al., 1982. The sequences for NT and ACR are shown in Fig. 1.

Results and Discussion

One of the most important forces involved in protein interactions is called the hydrophobic interaction force. Other forces which also play a role are electrostatic, van der Waals, and hydrogen bonding. The hydrophobic interaction force is poorly understood but is known to affect the entropy of the solvent and the solute (protein). Yet this force is so important in explaining why some proteins are soluble in water and polar solvents or lipids and nonpolar solvents. The hydrophilicity index is a method of quantifying this important force.

The plot of the hydrophilicity index versus sequence position for Lapemis toxin is shown in Fig. 2. The overall molecule is quite hydrophilic as shown by the large positive area under the curve. Loop 1 occurs from sequence positions 4 through 15. Loop 2 occurs from sequence positions 24 through 37. Loop 3 occurs from sequence positions 42 through 51. Note that the loops 1, 2, and 3 regions are hydrophilic with the maximum hydrophilic values shown in loop 2.

The NT structure proposed is based on X-ray diffraction studies on toxin a from Laticauda semifasciata venom, which is another short-chain neurotoxin with similar sequence homology to the Lapemis toxin (Tsernoglou and Petsko, 1976). The proposed structure is shown in Fig. 3. In this figure the 60 amino acid residues are shown with the sequence number. The stippled circles represent hydrophilic residues based on the hydrophilicity index analysis. It has been shown that the tryptophan 27 residue is functionally important in the toxic role of Lapemis toxin (Tu and Hong, 1971). Studies on other short-chain neurotoxins have shown an importance to toxicity of glutamic acid 19 (Chang et al., 1971a), lysine 45 (Chang et al., 1971b), histidine 30 (Huang et al., 1972), and arginine 31 (Yang et al., 1974). From this information and the following information about the ACR, a proposal of the ACR-NT interaction is made.

The ACR is diagrammatically shown in Fig. 4 (Hayashi and Nomoto, 1986). The two α-subunits are the sites of acetylcholine or neurotoxin binding to the ACR. The α-subunit is proposed to go in and out of the cell membrane several times with the N-terminal portion being found on the extracellular side of the resident cell and the C-terminal portion being found in the cytosol or interior of the cell.

The plot of the hydrophilicity index versus sequence position for the α-subunit of the ACR was plotted in four sections with sequences 1 through 110 shown in Fig. 5; sequences 110 through 220 shown in Fig. 6; sequences 220 through 330 are shown in Fig. 7; and sequences 330 through 437 are shown in Fig. 8. The plots show many regions of hydrophilicity and regions of hydrophobicity. Intramembraneous regions M6, M7, M1, M2, and M3 are bracketed.

Hydrophilic regions are most likely found in the cytosol or extracellular regions due to the water environment. The hydrophobic regions are most likely found either in the hydrophobic environment of the cell membrane lipid bilayer or are internalized in globular domains of the protein, folding in such a way as to minimize contact with the water environment of the extracellular region or cytosol.

From the hydrophilicity index analyses and information about Lapemis toxin, other short-chain neurotoxins and the α-subunit of the ACR, a model of NT-ACR interaction is made and shown in Fig. 9. Information about the ACR α-subunit is tabulated in Table 2, which is used to make the proposed model. The carbohydrate moiety at asparginine 141 of the α-subunit of the ACR was determined by Nomoto (1986). Tryptophan 27 of the NT is important to toxicity and the proposed interaction is with the first N-acetylglucosamine of the carbohydrate moiety at the asparginine 141 of the ACR α-subunit. This interaction is based on the known interaction of the tryptophan residues of lysozyme in binding the N-acetylglucosamine of the bacterial membrane carbohydrate moieties (Zubay, 1983). The arginine 31 residue of the NT is important, probably due to

its chemical and structural similarity to acetylcholine. The lysine 45 and glutamic acid 19 of the NT probably play a role by charge interaction with complementary residues of the ACR α-subunit. The histidine 30 of the NT probably plays a role by aiding in stabilizing the interaction of the arginine 31 of the NT with the binding at or near the disulfide cystine 192-193 of the ACR α-subunit.

The exact nature of the binding and specificity of the binding are still unclear and certainly lies in similar complementary regions of the NT and ACR α-subunit. The hydrophilicity index analysis has shown the hydrophilic nature of the NT and suggests the NT would seek out a hydrophilic region of the α-subunit of the ACR.

The more studies concentrated on the ACR-NT interaction will lead to the exact chemical nature of binding, giving a clearer picture of the mechanism of toxicity of NT. This work will also aid in the understanding of the structure-function relationships of the α-subunit ACR and the entire intact ACR, which will aid in treatments of pathological disorders of the ACR such as found in snakebites and myasthenia gravis.

Table 1. Assigned Hydrophilicity Values

Amino Acid		Hydrophilicity Values*	s,kcal/mcl**
R	Arginine	3.0	3.0
D	Aspartic acid	3.0	2.5
E	Glutamic acid	3.0	2.5
K	Lysine	3.0	3.0
S	Serine	0.3	0.3
N	Aspargine	0.2	0.2
Q	Glutamine	0.2	0.2
G	Glycine	0.0	0.0
P	Proline	0.0	-1.4
T	Threonine	-0.4	-0.4
A	Alanine	-0.5	-0.5
H	Histidine	-0.5	-0.5
C	Cysteine	-1.0	-1.0
M	Methionine	-1.3	-1.3
V	Valine	-1.5	-1.5
I	Isoleucine	-1.8	-1.8
L	Leucine	-1.8	-1.8
Y	Tyrosine	-2.3	-2.3
F	Phenylalanine	-2.5	-2.5
W	Tryptophan	-3.4	-3.4

*Hopp and Woods, 1981.

**Solvent parameter values assigned by Levitt (1976). The values of the hydrophobic solvent parameters s are taken as the measured free energy of transfer from water to ethanol in kcal/mol. The values range from +3 to -3.4 with hydrophilic residues indicated by positive values and hydrophobic residues indicated by negative values.

Table 2. Assignment of Sequence Information for the Acetylcholine Receptor α-Subunit.

Sequence #	Assignment	Reference #
1 - 141	Extracellular	1
141	Site of glycosylation	2,5
142 - 151	Intramembraneous M6	1
152 - 158	Cytosolic	1
159 - 191	Intramembraneous M7	1
182 - 198	Neurotoxin binding region, also binds antibodies	3
185 - 196	Binds α-bungarotoxin	4
192 - 193	ACH binding site	2
192 - 210	Extracellular	1
211 - 236	Intramembraneous M1	1
237 - 242	Cystolic	1
243 - 260	(Intramembraneous) (M2)	1
261 - 276	(Extracellular)	1
277 - 297	(Intramembraneous) (M3)	1
298 - 329	(Cystolic)	1
330 - 437	Cystolic	1

Ref. 1 - Ratnam et al., 1986
Ref. 2 - Oblas et al., 1986
Ref. 3 - Mulac-Jericevic and Atassi, 1986
Ref. 4 - Neuman et al., 1986
Ref. 5 - Nomoto et al., 1986

Lapemis hardwickii Lapemis Toxin Sequence

```
         10        20        30        40        50        60
MTCCNQQSS.QPKTTTNCAE.SSCYKKTWSD..IRGTRIERGC.GCPQVKPGIK.LECCHTNECN.N
```

Torpedo californica Acetylcholine Receptor (α-subunit) Sequence

```
          10        20        30        40        50        60
 SEHETRLVA.NLLENYNKVI.RPVEHHTHFV.DITVGLQLIQ.LISVDEVNQI.VETNVRLRQQ.WIDVRLRWNP

70        80        90        100       110       120       130
ADYGGIKKIR.LPSDDVWLPD.LVLYNNADGD.FAIVHMTKLL.LDYTGKIMWT.PPAIFKSYCE.LIVTHFPFDQ

140       150       160       170       180       190       200
QNCTMKLGIW.TYDGTKVSIS.PESDRPDLST.FMESGEWVMK.DYRGWKHWVY.YTCCPDTPYL.DITYHFIMQR

210       220       230       240       250       260       270
IPLYFVVNVI.IPCLLFSFLT.GLVFYLPTDS.GEKMTLSISV.LLSLTVFLLV.IVELIPSTSS.AVPLIGKYML

280       290       300       310       320       330       340
FTMIFVISSI.IITVVVINTH.HRSPSTHTMP.QWVRKIFIDT.IPNVMFFSTM.KRASKEKQEN.KIFADDIDIS

350       360       370       380       390       400       410
DISGKQVTGE.VIFQTPLIKN.PDVKSAIEGV.KYIAEHMKSD.EESSNAAEEW.KYVAMVIDHI.LLCVFMLICI

420       430       437
IGTVSVFAGR.LIELSQEG
```

The single letter notation for the twenty amino acid residues are: A - Alanine, C - Cysteine/Cystine, D - Aspartic acid, E - Glutamic acid, F - Phenylalanine, G - Glycine, H - Histidine, I - Isoleucine, K - Lysine, L - Leucine, M - Methionine, N - Aspargine, P - Proline, Q - Glutamine, R - Arginine, S - Serine, T - Threonine, V - Valine, W - Tryptophan, and Y - Tyrosine.

Fig. 1. Lapemis hardwickii Lapemis Toxin Sequence and Torpedo californica Acetylcholine Receptor (α-subunit) Sequence.

Lapemis hardwickii Lapemis toxin

Hydrophilicity Analysis

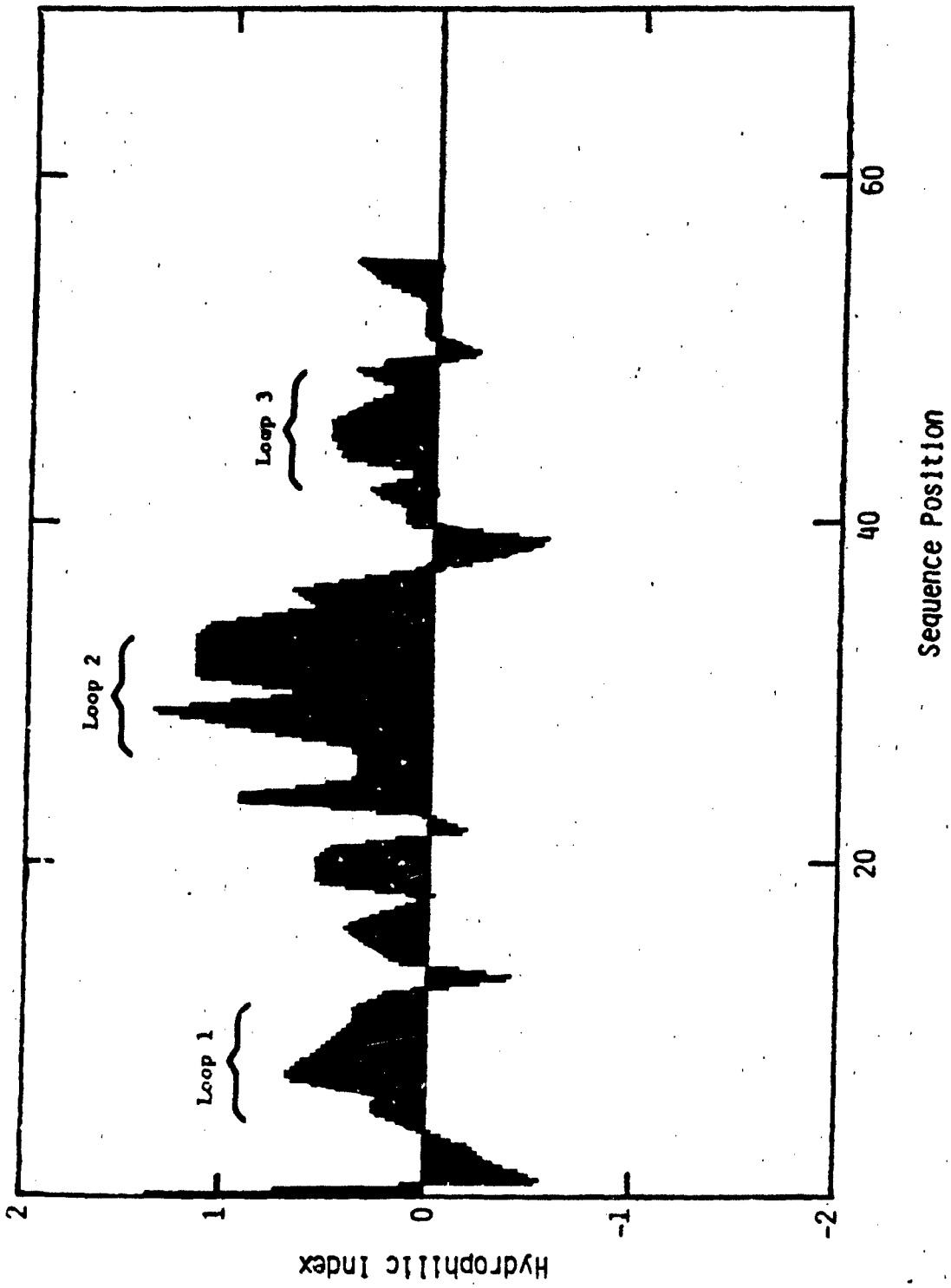
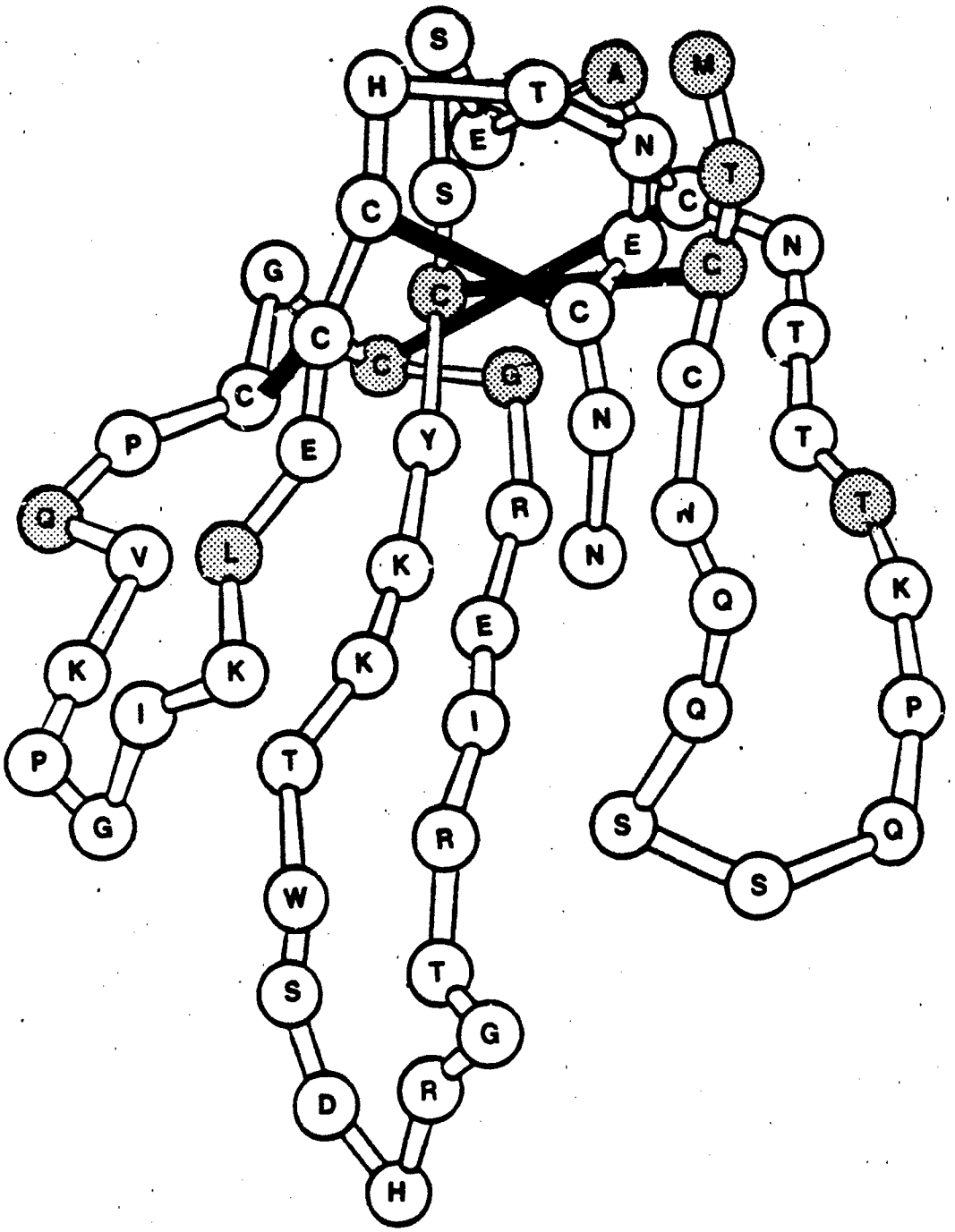


Fig. 2

Proposed Structure of Lapemis Toxin
Lapemis hardwickii Lapemis Toxin

10 20 30 40 50 60

MTCCNQQSS.QPKTTTNCAE.SSCYKKTWSD.ERGTRIERGC.GCPQVKPGIK.LECCHTNECN.N



Stippled circles are hydrophilic residues based on the hydrophilicity analysis.

Fig. 3

Diagrammatic Model of the
Acetylcholine Receptor

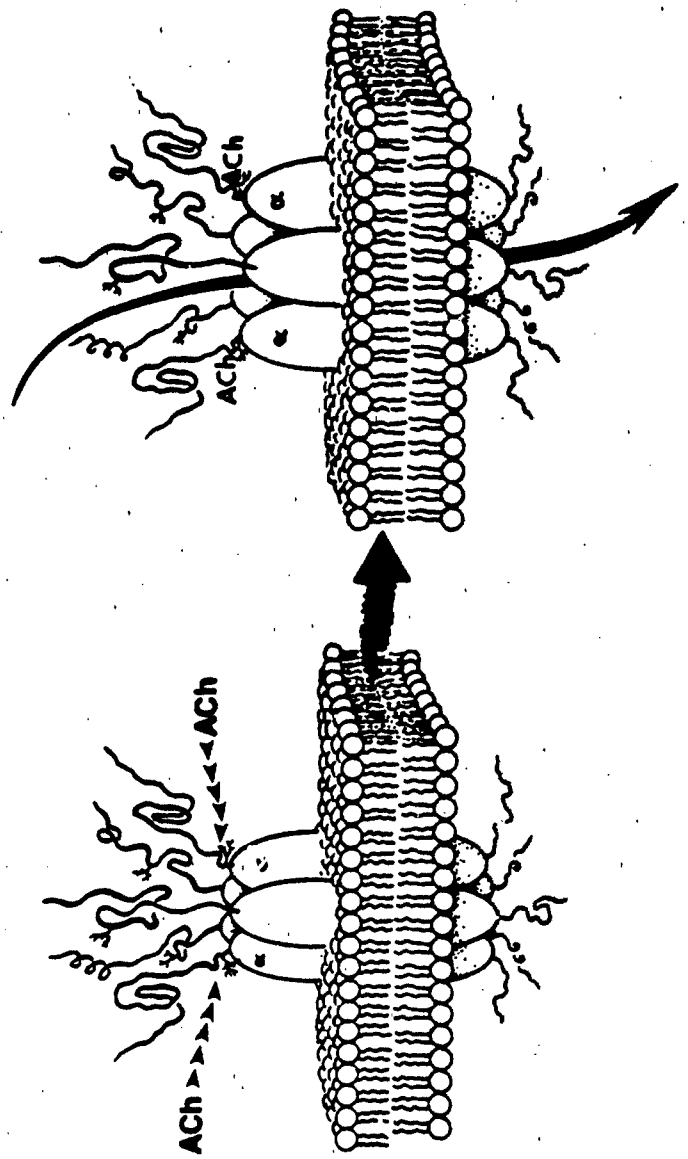


Fig. 4

Acetylcholine Receptor Alpha-
Subunit Hydrophilicity Analysis
on Sequence Positions 1 - 110

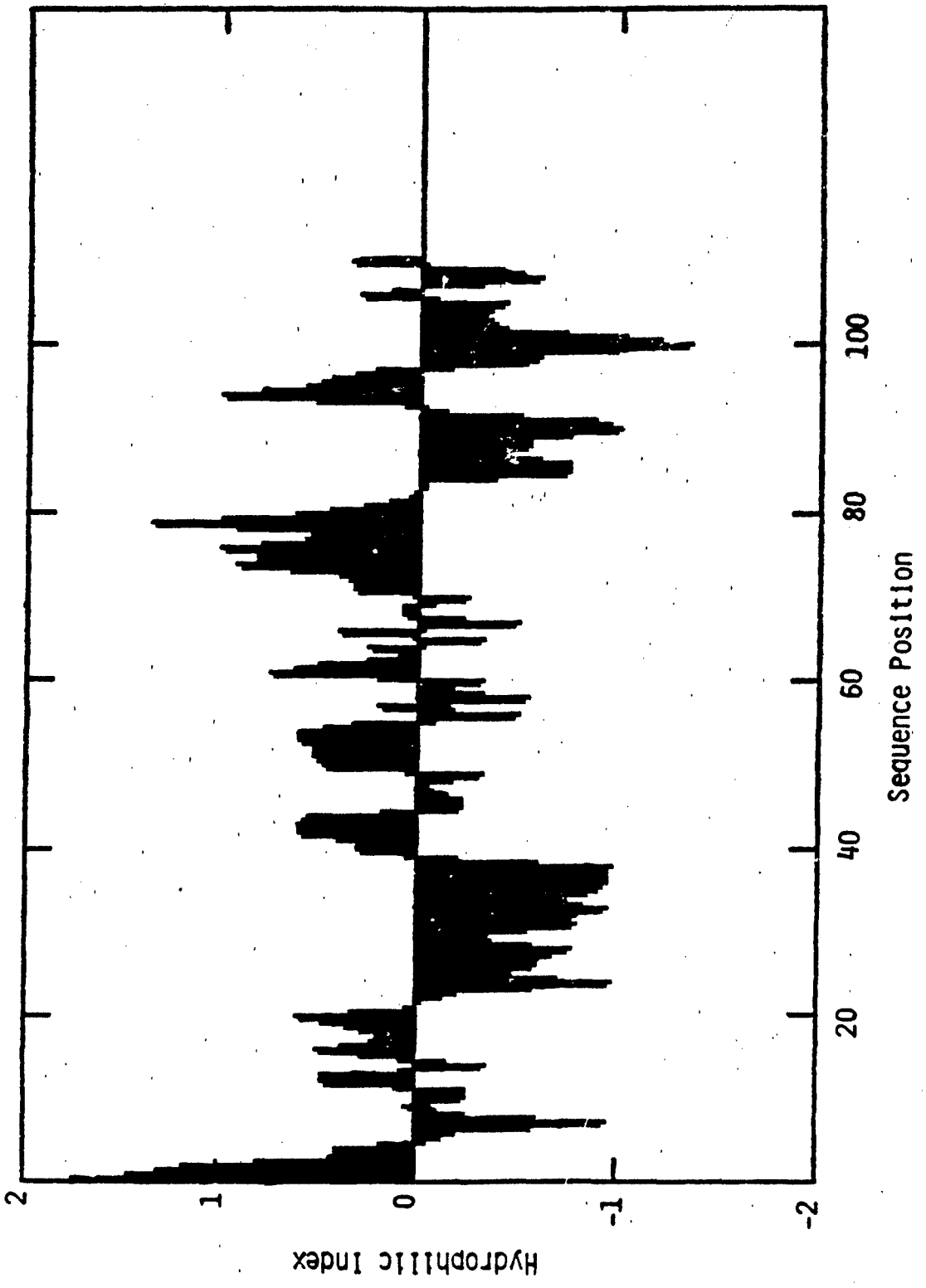


Fig. 5

Acetylcholine Receptor Alpha-Subunit Hydrophilicity Analysis on Sequence Positions 110 - 120.

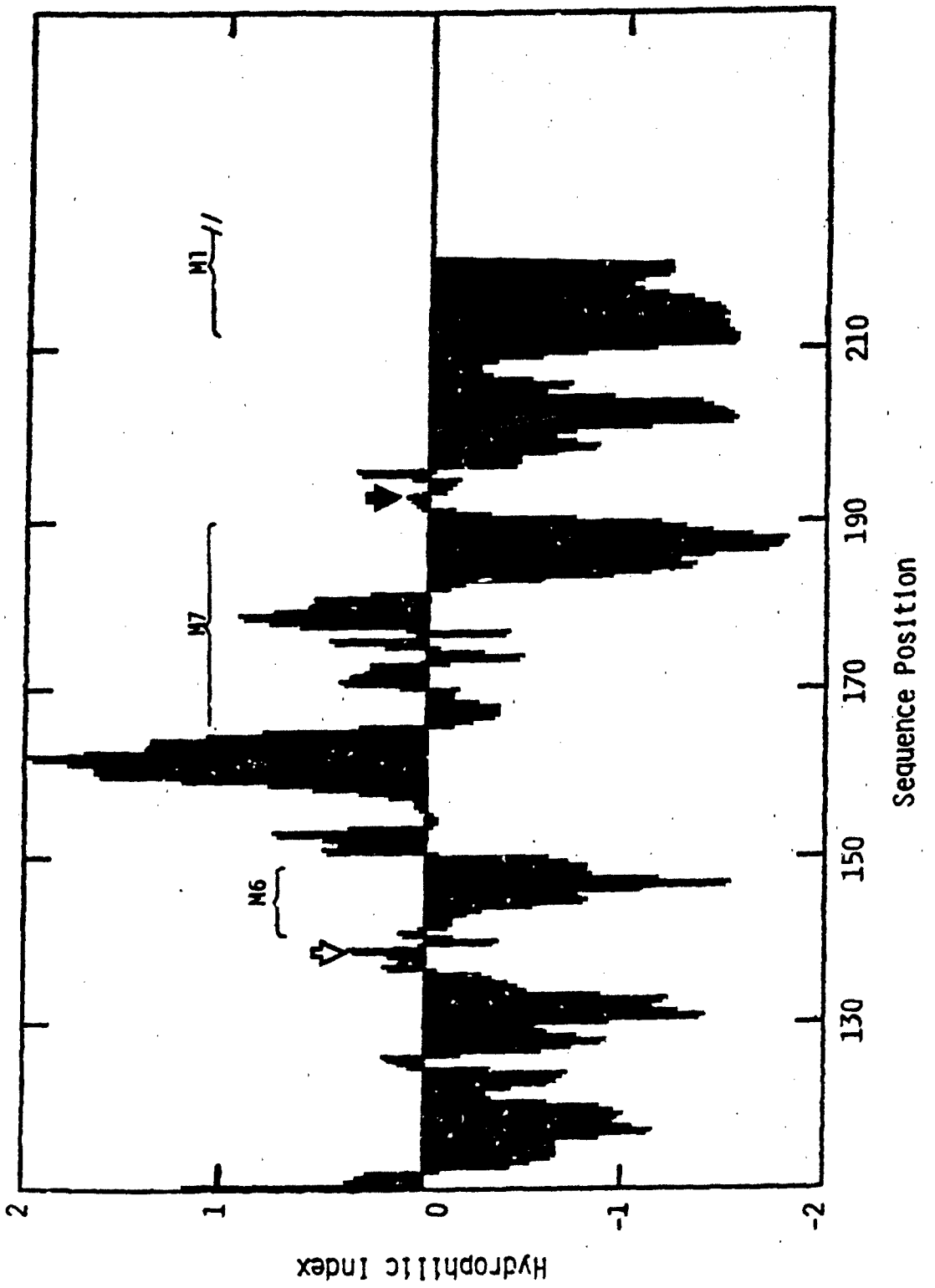


Fig. 6

Acetylcholine Receptor Alpha-Subunit Hydrophilicity Analysis on Sequence Positions 220 - 330.

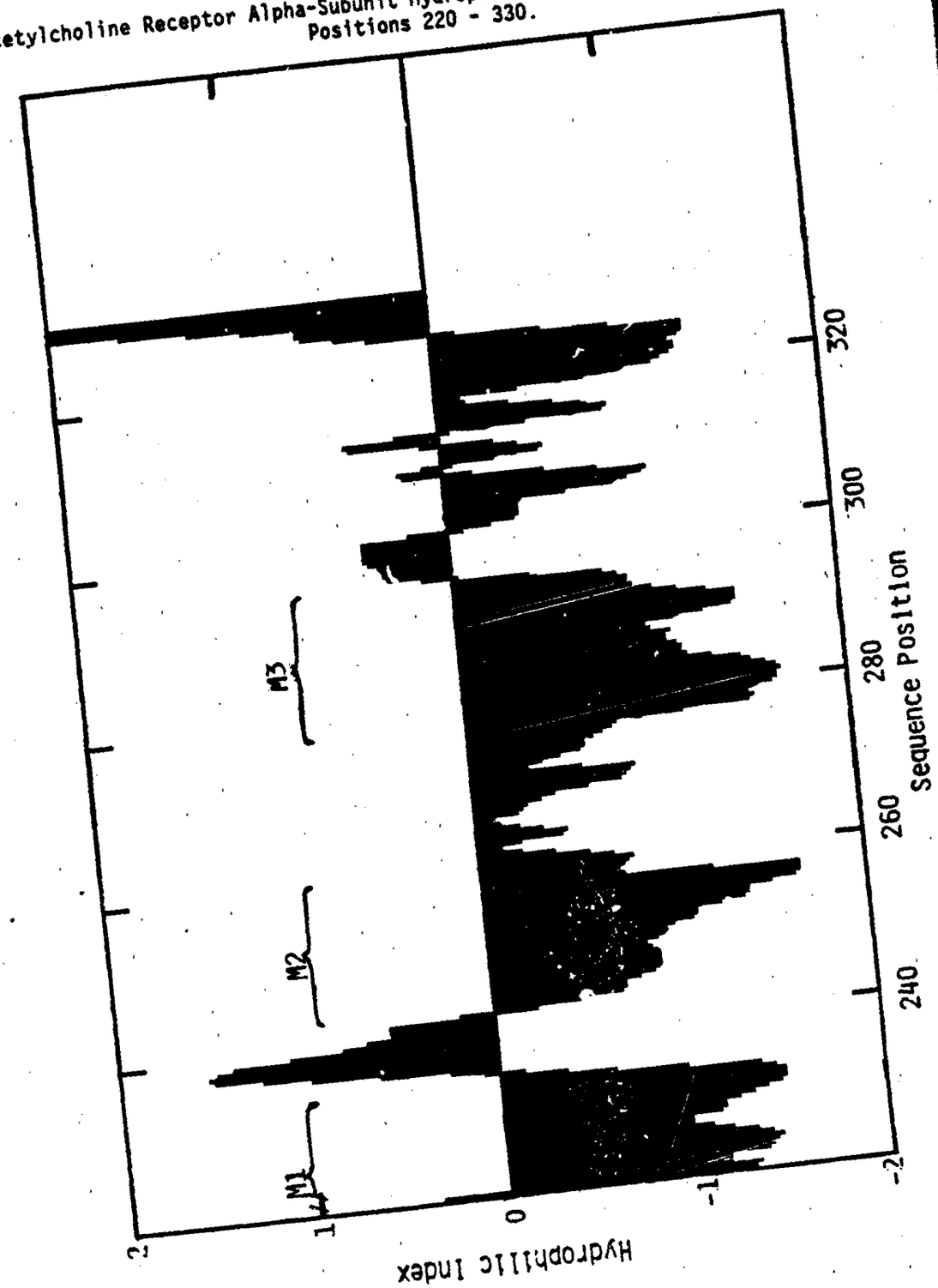


Fig. 7

Acetylcholine Receptor Alpha-Subunit Hydrophilicity Analysis on Sequence Positions 330 - 437.

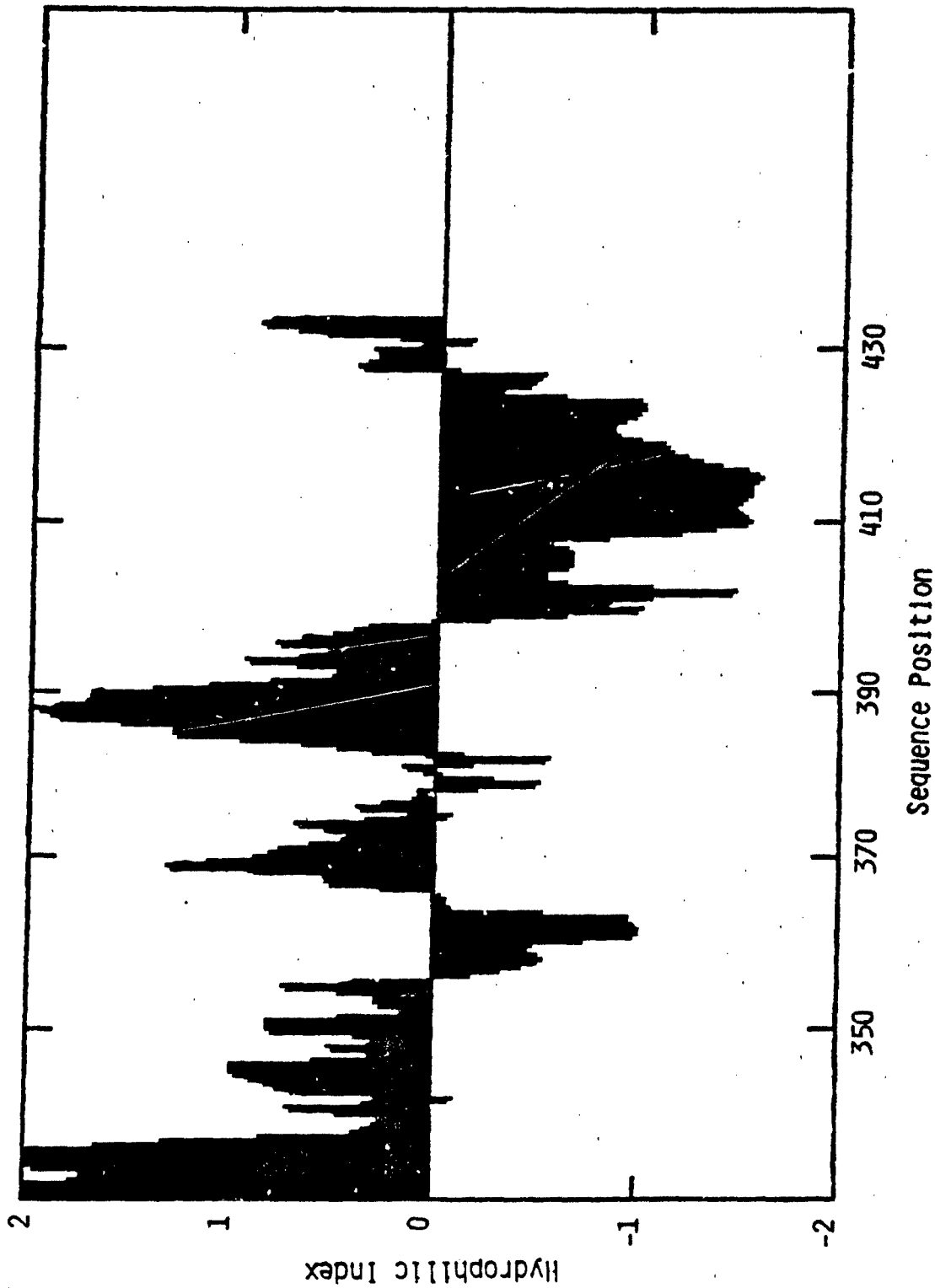


Fig. 8

Model of NT - Alpha ACR Interaction

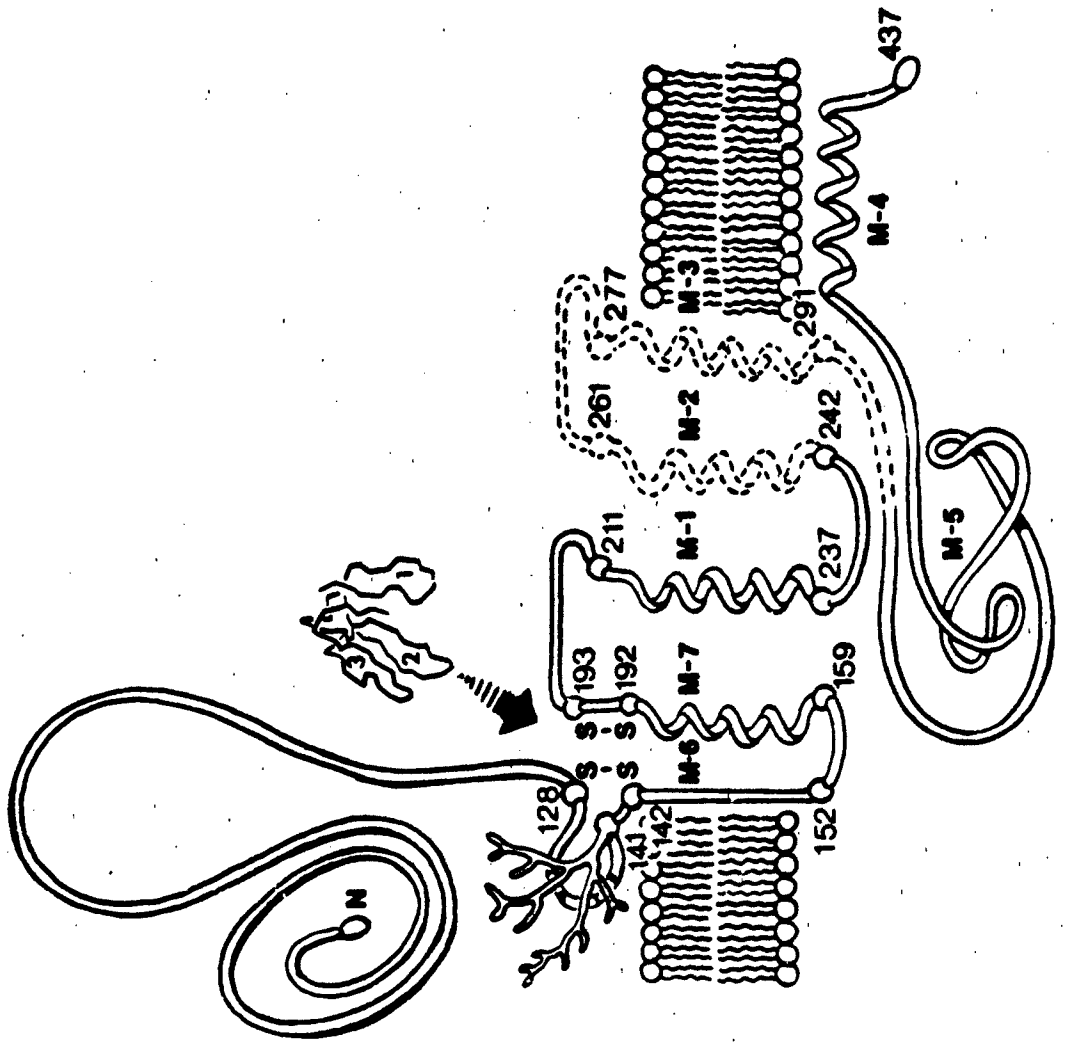


Fig. 9

B. Isolation and Chemical Modification of Lapemis Toxin from Lapemis hardwickii Toxin by Anthony T. Tu and Nobuhiro Mori

Methods

Sea snakes, Lapemis hardwickii, were captured in the Gulf of Thailand near Songkla, Thailand. In order to extract the venom, the sea snakes were decapitated as soon as the fishermen brought them to the Songkla harbor, and the venom glands were excised. After dry venom glands were received in Colorado from Thailand by air mail, they were pulverized thoroughly with an electric grinder and the venom was extracted with distilled water. After the insoluble tissue debris was removed by centrifugation, the supernatant liquid was filtered and lyophilized.

Lapemis Toxin: Lapemis toxin was isolated from crude venom essentially by the method of our earlier publication (Fox et al., 1977). Two grams of venom were dissolved in 5 ml of 10 mM potassium phosphate, pH 6.5 buffer containing 0.1 M NaCl. Upon dissolution, the mixture was loaded onto a Sephadex G-50 column (2.5 x 100 cm) equilibrated with the phosphate buffer. The elution was carried out with the phosphate buffer at 4°C; three ml fractions were collected.

The absorbance at 280 nm of each fraction was determined with a spectrometer. The toxicity of each pooled peak was analyzed according to the method of Litchfield and Wilcoxon (1949). Various quantities of the lyophilized protein peaks were first dissolved in a 0.09% NaCl solution. Swiss Webster white mice weighing 18-20 g were then injected with 0.1 ml of the toxin solution in the tail vein and segregated for 24 h to monitor the effects. Most of the mice either succumbed to the toxin injection within 60 min or survived the 24 h interval.

The G-50 peak with the highest specific toxicity was dissolved in 4 ml of distilled water, and dialyzed (Spectra/Por dialysis membrane, MWCO 2,000) against 10 mM potassium phosphate, pH 7.8, buffer (2-4°C). This sample was then applied to a carboxymethyl cellulose (Sigma Chemical) cation-exchange column (2 x 35 cm) equilibrated with the same buffer at 2-4°C. The column was developed with a linear salt gradient from 0 to 0.4 NaCl, 1000 ml total volume. The absorbance at 280 nm was monitored and the protein peaks pooled and assayed for toxicity.

Isolation of ACR: ACR-rich membrane and pure ACR were isolated from the electric organ tissue of Torpedo californica. ACR-rich membrane is the membrane fragment containing ACR; therefore, it also contains phospholipids. Purified ACR is composed of five subunits of four kinds having stoichiometry $\alpha_2\beta\gamma\delta$. ACR was isolated by the affinity chromotographic method of Froehner and Rafto (1979). Cobrotoxin affinity resin

was prepared as previously described (Brockes & Hall, 1975), using Sepharose 4B. The amount of active α-neurotoxin per ml of Sepharose was estimated to be approximately 0.1 mg or about 20% of the input. ACR-rich membrane was isolated by the method of Froehner and Rafto (1979).

All procedures were carried out at 4°C. Approximately 75 g of frozen tissue was sliced into small pieces with a razor blade and homogenized in 250 mL of 10 mM Tris-Cl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1 mM $PhCh_2SO_2F$, and Trasylol (10 units/mL) (buffer A) in a Waring blender. The homogenate was passed through a double layer of cheesecloth and centrifuged for 45 min at 27,000 g. The pellet was resuspended in 25 mL of buffer A with a Dounce homogenizer, and Triton X-100 was added to a final concentration of 1%. After 1 h, insoluble material was removed by centrifugation (30 min at 27,000 g), and the supernatant (Triton extract) was retained for affinity purification.

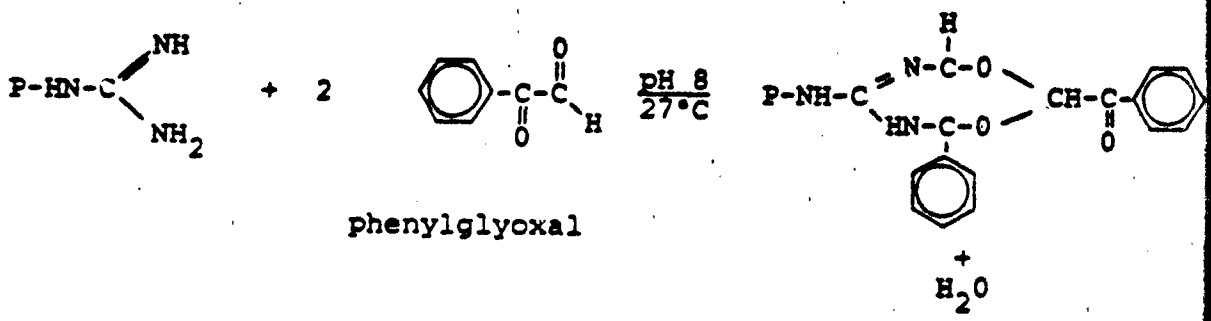
Pure ACR was isolated as follows: The Triton extract was stirred with 2-3 mL of cobrotoxin-Sepharose 4B (previously washed with buffer A containing 1 M NaCl and then equilibrated with buffer A) for 1.5-2 h. The mixture was then poured into a 10-mL plastic syringe with a nylon net support to form a column. The column was washed sequentially with 5 mL of buffer A containing 1.0% Triton X-100 (buffer B), 10 mL of buffer B containing 1 M NaCl, and 5 mL of buffer A containing 0.1% Triton X-100. Approximately 0.8 column volume of 1 M carbamoylcholine chloride in buffer A containing 0.1% Triton X-100 was run into the column. After 0.5-2 h, the first elution was collected. A second elution was collected after a further incubation of 12-15 h or, alternatively, the column contents were poured into 20-25 mL of carbamoylcholine solution and stirred slowly for 12-15 h. Eluted receptor was then collected after repouring the column. Both procedures for the second elution give comparable results. The affinity-purified fractions were pooled and then dialyzed against four changes of 500 mL of buffer A containing 0.1% Triton X-100 for at least 2 h each. For some preparations, 10 mM NEM was added to all buffers except the dialysis buffer which contained 1 mM NEM. Purified receptor stored at 4°C showed no loss of [^{125}I]-αBuTx binding activity and, except for some aggregation, no substantial change in its subunit structure over a period of several months. Receptor stored at -70°C lost some binding activity after several freezings and thawings, but no change in subunit structure was noted.

Modification of Arginine Residues: Modification of arginine residues in Lapemis toxin with phenylglyoxal was performed by a modification of the method described by Yang et al. (Biochim. Biophys. Acta 365, 1-14, 1974).

To a solution of Lapemis toxin (1.6 μmoles) in 0.1 ml of 0.2 M N-ethylmorpholine acetate buffer (pH 8.0), a 100-fold molar excess of phenylglyoxal in 0.3 ml of the same buffer

was added, and the reaction was allowed to proceed at 27°C for 1 h. Reactions at pH values other than 8.0 were carried out in the same way with appropriate buffers. The mixture was passed through a column of Bio-Gel P-6 (0.9 x 40 cm, MW cut off 6000 daltons) followed by ion exchange chromatography on CM-cellulose with a gradient of increasing salt concentration from 0.005 M ammonium acetate, pH 5.8 to 6.8. The fractions of the main protein peaks were lyophilized and desalted by using a Bio-Gel P-6 column equilibrated with 1% acetic acid. The protein fractions were then pooled and lyophilized.

The homogeneity of the modified toxin was checked by HPLC. Samples of phenylglyoxal derivatives were applied on an ODS column (4.6 mm x 25 cm) equilibrated with 0.1% TFA (buffer A) and eluted with a linear gradient of 40% buffer B (0.1% TFA containing 30% acetonitrile and 70% 2-propanol) for 40 min. Flow rate was 0.8 ml/min and the effluent is monitored at 280 nm.



phenylglyoxal

Phenylglyoxal derivatives were very stable even after HCL hydrolyzation.

Results and Discussion

Fractionation - The fractionation patterns of the first and second steps are shown in Fig. 10A and B.

Homogeneity - Homogeneity was examined by analytical HPLC, Beckman series 345, and found to be a single peak. The toxin was also homogeneous in gel electrophoresis.

Toxicity - The final purified toxin was lethal at the level of 0.15 µg/g by intravenous injection in mice. In order to minimize the use of mice, the LD_{50} of crude venom was 1.4 µg/g, while that of purified Lapemis toxin was found to be 0.66.

Purified ACR and Binding to Lapemis Toxin

Purified ACR is composed of five subunits, of which two are identical, giving the stoichiometry $\alpha_2\beta\gamma\delta$. Since two of the subunits are identical, ACR showed four bands in SDS gel electrophoresis (Fig. 11). Lapemis toxin bound to ACR and formed a complex (Fig. 12).

ACR-Lapemis toxin complex dissociated into ACR and Lapemis toxin after reduction by mercapto ethanol (Fig. 12). SDS alone did not dissociate the complex (Fig. 12).

Lapemis toxin replaced α-bungarotoxin (α-BTx) in binding to ACR, indicating it is a competitor to ^{125}I-α-BTx and binds to the same site in ACR.

Modification of Arginine Residue: There are three arginine residues out of 60 total residues. An objective of our research is to find which residues are involved in the ACR binding. In order to clarify this, two experiments are needed. One is a control experiment; that is to modify arginine residues of free Lapemis toxin (not bound to ACR). The other experiment is to modify Lapemis toxin after binding to ACR. The first experiment was done and it was found that two arginines out of three were modified for unbound Lapemis toxin (Table 2). Chemical modification of Lapemis toxin bound to ACR has not been done yet.

Table 2. Amino Acid Composition of Lapemis Toxin (Unbound to ACR) Before and After Modification of Arginine Residue.

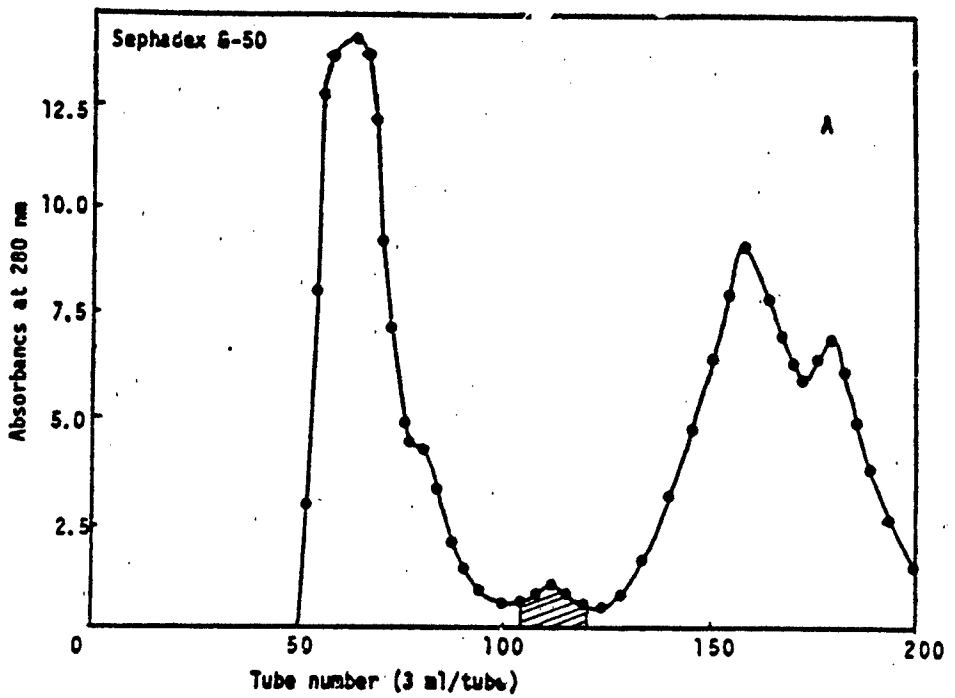
Amino Acids	Before Modification (from Sequence)	After Modification (Actual Analysis)	
Asp	6	5.6	(6)
Glu	8	8.5	(9)
Ser	5	4.6	(5)
Gly	4	4.4	(4)
Thr	7	7.3	(7)
His	2	1.9	(2)
Ala	1	1.3	(2)
Pro	3	3.3	(3)
Arg	3	1.4	(2)
Tyr	1	1.0	(1)
Met	1	0.5	(1)
Val	1	1.3	(1)
Cys	9	8.5	(9)
Ile	2	2.4	(2)
Leu	1		
Lys	5	4.4	(4)
Phe	0	0	
Trp	1	-	
Total	61		

LEGEND

Fig. 10 Isolation scheme of the Lapemis toxin from the venom of Lapemis hardwickii. The isolation procedure is described under Experimental Method.

Fig. 11 Subunits of reduced acetylcholine receptor shown in SDS polyacrylamide gel electrophoresis.

Fig. 12. SDS-electrophoresis of 1, 2, 3, indicating the dissociation of ACR-LT complex to individual subunits and LT (Lapemis toxin) after 2-mercaptoethanol reduction. 4, 5, 6 indicated that ACR-LT complex is not dissociated without 2-mercaptoethanol. See very weak LT band comparing to the electrophoretic band of 1, 2, 3.


Sephadex G-50
A
Absorbancs at 280 nm
12.5
10.0
7.5
5.0
2.5
0
50
100
150
200
Tube number (3 ml/tube)

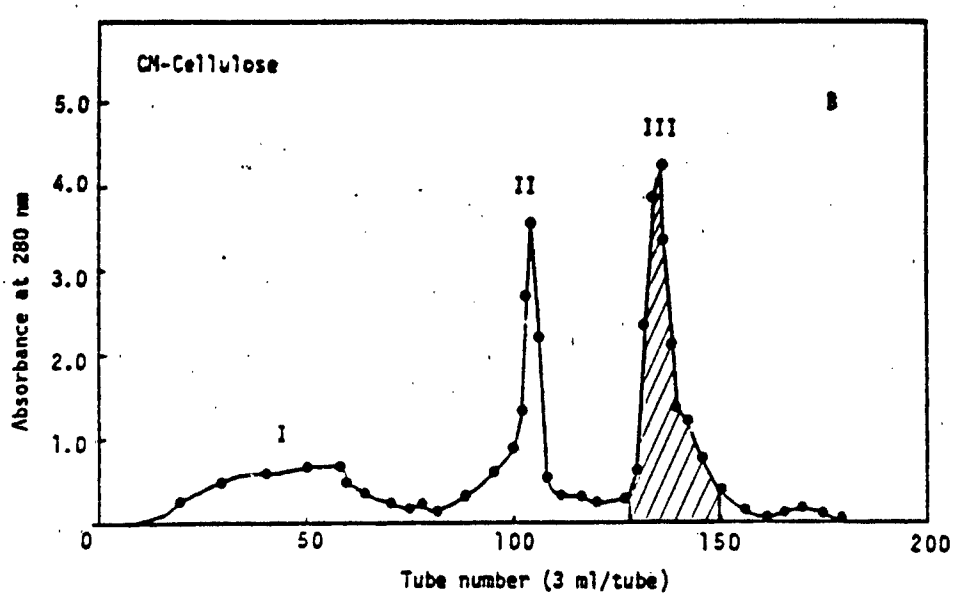

CM-Cellulose
B
I
II
III
Absorbance at 280 nm
5.0
4.0
3.0
2.0
1.0
0
50
100
150
200
Tube number (3 ml/tube)

Fig. 10

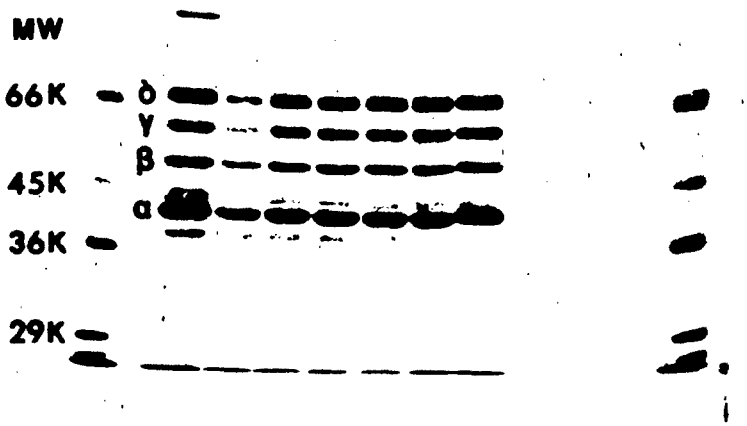


Figure 11

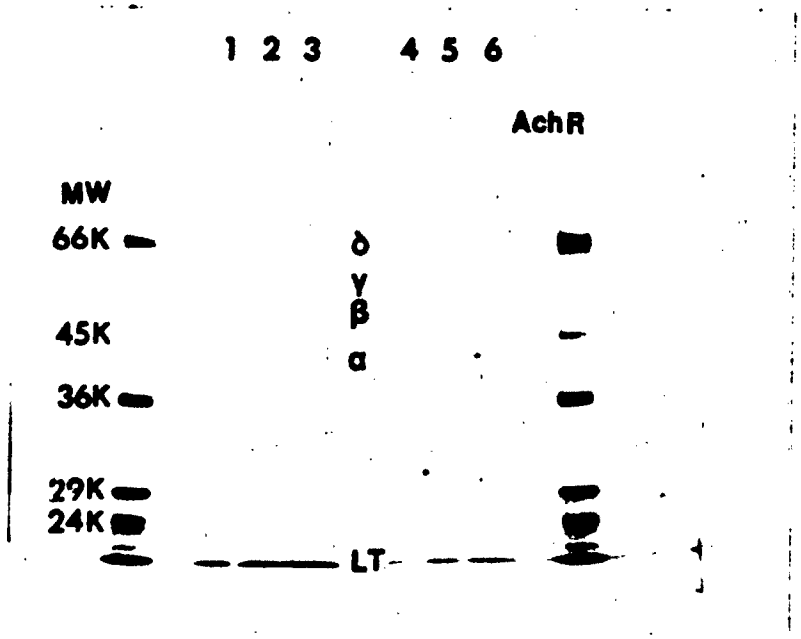


Figure 12

C. Isolation and Amino Acid Sequence of Neurotoxin from the Venom of Sea Snake Acalyptophis peronii

by Anthony T. Tu and Nobuhiro Mori

Experiments

Acalyptophis peronii was captured in the Gulf of Thailand off the coast of Songkla in November-December 1979. The sea snake was decapitated and venom glands were removed. Dried venom glands were sent to Colorado and extracted twice with distilled water after venom glands were pulverized. The extracted proteins were designated "crude venom." Lyophilized crude venom was stored in a freezer until used.

Isolation of Acalyptophis Toxin

The major toxin, Acalyptophis toxin, was isolated from crude venom from Acalyptophis peronii using two-step column chromatography of G-50 Sephadex and CM-cellulose. The condition for the isolation was the same as the method of Laticauda toxin isolation published by Tu et al. (1971).

Homogeneity was established by LKB Tachophor model 2127 using a cationic system. Cacodylic acid at pH 7.0 was used as the leading ion and 0.01 M creatinine as the terminating ion. Ampholine (1%) was used as a spacer electrolyte. A constant current of 75 μA and a variable voltage of 3-12 kV were used.

Amino Acid Sequence Determination

The purified toxin was reduced and alkylated following the method of Crestfield as described by Elzinga (1970). Microsequence analysis was performed on approximately 500 pico moles of each peptide using an Applied Biosystems model 470A protein sequencer similar to that described by Hewick et al. (1981). Phenylthiohydantoin amino acids were identified by HPLC using a Beckman-Altex Ultrasphere ODS column and the trifluoroacetic acid/acetonitrile buffer system reported by Hawke et al. (1982).

For tryptic digestion, the toxin was first treated with citraconic anhydride to block the lysine residues. The citraconylated CM-toxin (CM-CA toxin) was then subjected to tryptic digestion using an enzyme to substrate ratio of 1:30 (w/w). After 16 h at 23°C the digestion was terminated by the addition of soybean trypsin inhibitor and glacial acetic acid to 25%. The mixture was then passed through a Sephadex G-50 column equilibrated with 25% acetic acid which served to separate the products. Separation was made further by an HPLC reverse phase column.

Different toxin peptide fragments were made using the endopeptidase, arginine C, after carboxymethylation of the reduced toxin.

Results and Discussion

Isolation of Neurotoxin

The major toxin, Acalyptophis toxin, was isolated by a combination of gel filtration and CM-cellulose ion-exchange chromatography (Fig. 1A,B). Homogeneity was established by analytical capillary isotachophoresis LKB Tachophor model 2127 using cationic system. A sharp single peak was obtained for the toxin, indicating a high degree of homogeneity (Fig. 1C). Toxicity, expressed as LD_{50}, was 0.125 µg/g in mice by intravenous injection.

Amino Acid Sequence

Three methods were used to determine the amino acid sequence of Acalyptophis toxin. First, the sequence analysis was made directly using carboxymethylated toxin. The second method was to analyze the fragments obtained from tryptic digestion. The third method was to analyze the sequence of endopeptidase, arginine C treated peptide fragments.

1. The sequence obtained from CM-toxin is shown here:

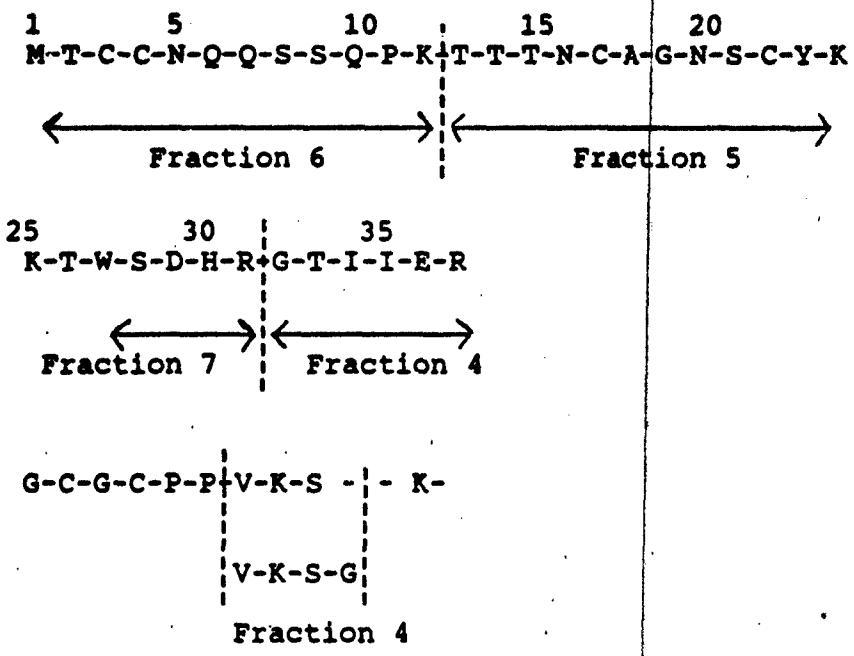
```
 1                  2                        10
Met-Thr-CMC-CMC-Asn-Gln-Gln-Ser-Ser-Gln-

 11                 15                       20
Pro-Lys-Thr-Thr-Thr-Asn-CMC-Ala-Gly-Asn-

 21                 25                       30
Ser-CMC-Tyr-Lys-Lys-Thr-Trp-Ser-Asp-Thr-

 31                 35
Arg-Gly-Thr-Ile-Ile-Glu-Arg-
```

2. The Sequence of Tryptic Digestion Fragments

Toxin after tryptic digestion was subjected to separation by HPLC. Altogether ten fractions were observed in the HPLC chromatogram (Fig. 2). Fractions 3, 4, 5, 6, and 7 were sequenced with the results shown below.

Fraction 3	G-C-G-C-P-Q-V-K-S - - K
Fraction 4	G-T-I-I-E-R and V-K-S-G
Fraction 5	T-T-T-N-A-G-N-S-Y-K
Fraction 6	M-T-C-C-N-Q-Q-S-S-Q-P-K
Fraction 7	S-Q-H-R

By combining the sequence data of fragments and carobxymethylated toxin, the following overlapping patterns can be obtained.



Trypsin used for fragmentation apparently is contaminated with chymotrypsin since W(27)-S(28) and Q-V bonds were hydrolyzed. Fraction 4 contained two peptides G(32)-T-I-I-E-R(37) and V-K-S-G - - , which were coeluted in the same HPLC fraction.

3. Endopeptidase Arg C Treated Peptide Fractions

Three main peaks 1, 2, and 3 were obtained (Fig. 3), which are being analyzed for their sequences. No data are available yet.

LEGEND

Fig. 13 Isolation scheme of the Acalyptophis toxin from the venom of Acalyptophis peronii (A and B). The isolation procedure is described under Experimental Method. Homogeneity was established by isotachophoresis using a cationic system (C).

Fig. 14 HPLC profiles of the tryptic digests of citraconylated CM-toxin.

Fig. 15 HPLC profiles of the endopeptidase Arg C digests of CM-toxin.

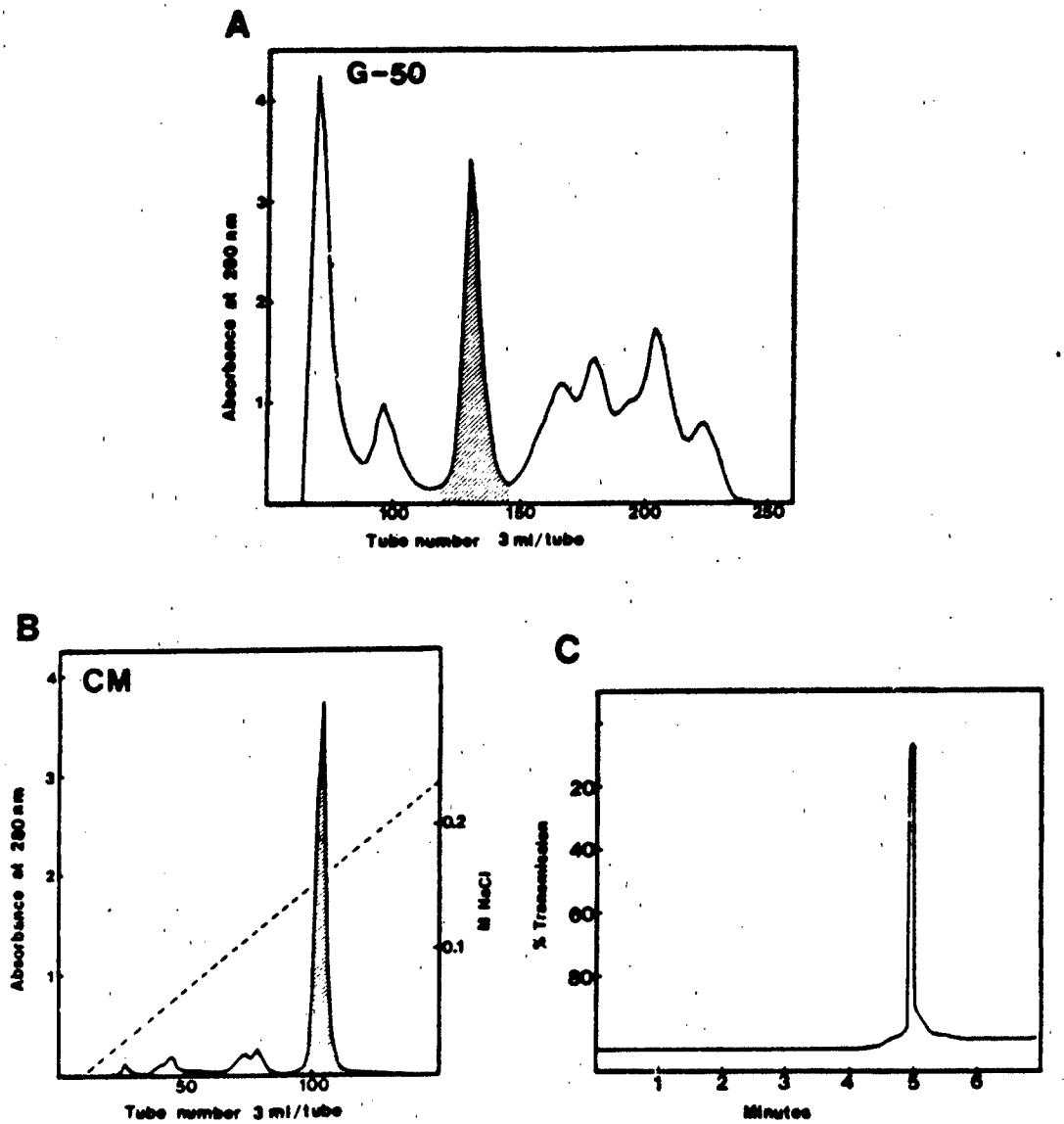

A
G-50
Absorbance at 280 nm
Tube number 3 ml/tube
B
CM
Absorbance at 280 nm
M NaCl
Tube number 3 ml/tube
C
% Transmission
Minutes

Fig. 13

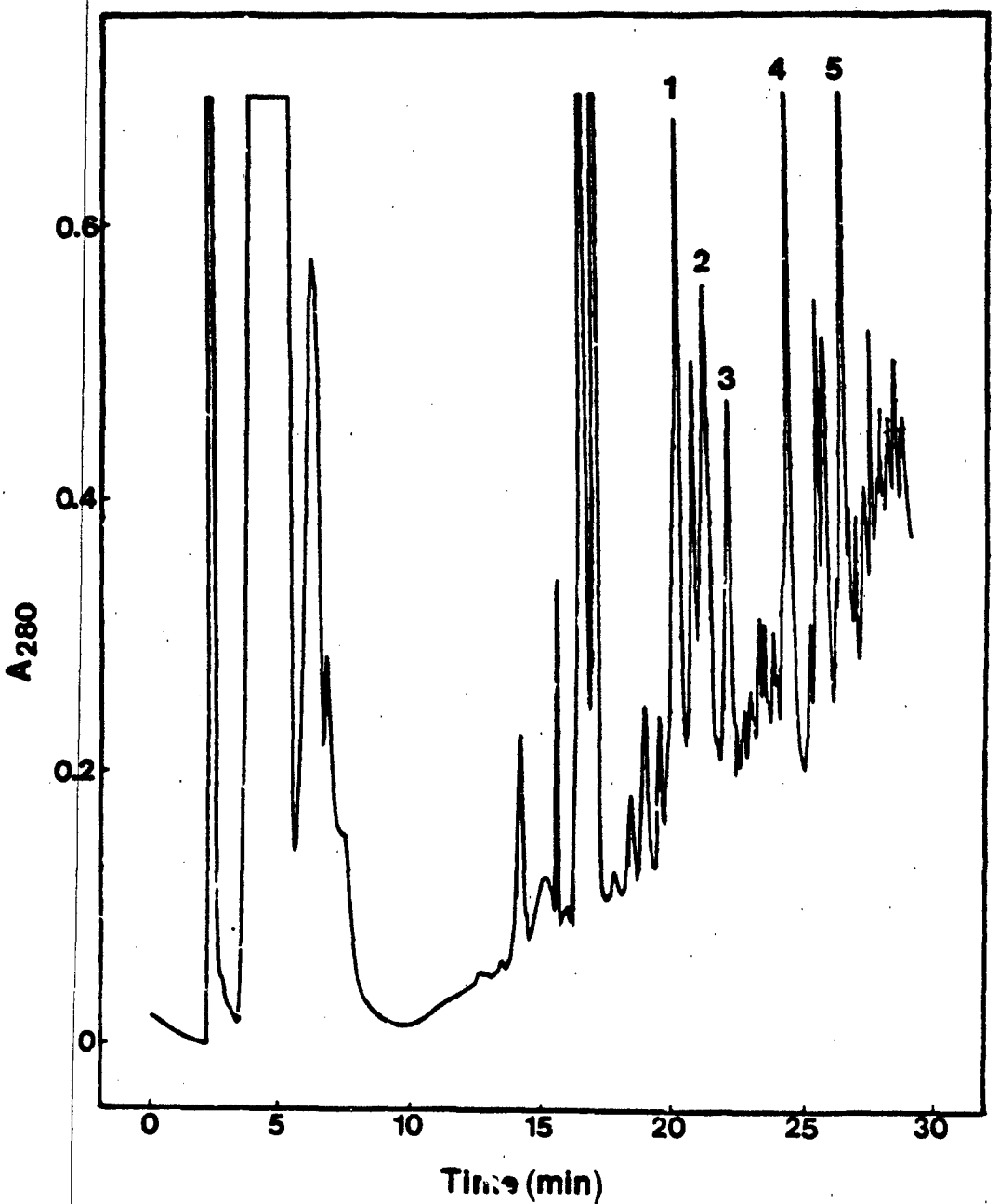


Fig. 14

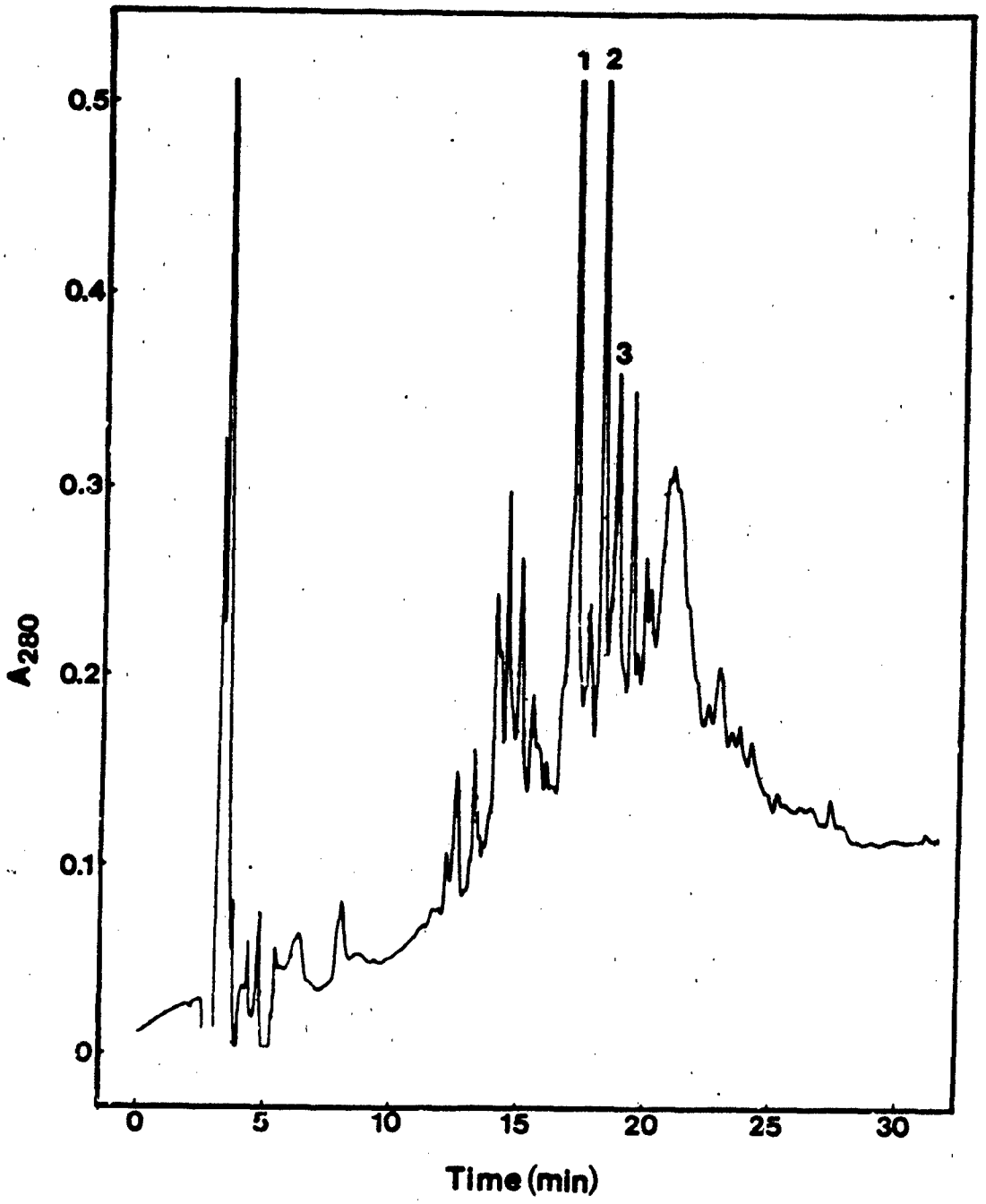


Fig. 15

References

Brockes, J. P., and Hall, Z. W. (1975). Acetylcholine receptors in normal and denervated rat diaphragm muscle. I. Purification and interaction with [^{125}I]-α-Bungarotoxin. Biochemistry 14, 2092-2099.

Chang, C. C., Yang, C. C., Kurobe, M., Nakai, K., and Hayashi, K. (1971a). The identification of the special glutamic acid residue essential for activity of cobrotoxin. Biochem. Biophys. Res. Commun. 43, 429.

Chang, C. C., Yang, C. C., Nakai, K., and Hayashi, K. (1971b). Studies on the status of free amino and carboxyl groups in cobrotoxin. Biochim. Biophys. Acta 251, 334.

Claudio, T., Ballivet, M., Patrick, J., and Heinemann, S. (1983). Nucleotide and deduced amino acid sequences of Torpedo californica acetylcholine receptor gamma chain. Proc. Natl. Acad. sci. 80, 1111-1115.

Elzinga, M. (1970). Amino acid sequence studies on rabbit skeleton muscle actin. Cyanogen bromide cleavage of the protein and determination of the sequences of seven of the resulting peptides. Biochemistry 9, 1365-1374.

Endo, T., Nakanishi, M., Furokawa, S., Joubert, F. J., Tamiya, N., and Hayashi, K. (1986). Stopped-flow fluorescence studies on binding kinetics of neurotoxins with acetylcholine receptor. Biochemistry 25, 395-404.

Fox, J. W., Elzinga, M., and Tu, A. T. (1977). Amino acid sequence of a snake neurotoxin from the venom of Lapemis hardwickii and the detection of a sulfhydryl group by laser Raman spectroscopy. FEBS Lett. 80, 217-220.

Froehner, S. C., and Rafto, S. (1979). Comparison of subunits of Torpedo californica acetylcholine receptor by peptide mapping. Biochemistry 18, 301-307.

Hawke, D., Yuan, P. M., and Shively, J. E. (1982). Microsequence analysis of peptides and proteins. Anal. Biochem. 120, 302-311.

Hayashi, K., and Nomoto, H. (1986). Relationship between structure and function of AChR. Med. Immunol. (Japan) 11, 425-434.

Hewick, R. M., Hunkapiler, H. W., Hood, L. E., and Dreyer, W. J. (1981). A gas-liquid solid phase peptide and protein sequenator. J. Biol. Chem. 256, 7990-7997.

Hopp, T. P., and Woods, K. R. (1981). Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. 78, 3824-3828.

Hopp, T. P., and Woods, K. R. (1983). A computer program for predicting protein antigenic determinants. Mol. Immunol. 20, 483-489.

Huang, J. S., Liu, S. S., Ling, K. H., Chang, C. C., and Yang, C. C. (1972). Photoxidation of cobrotoxin. J. Formosan Med. Assoc. 71, 383.

Levitt, M. (1976). A simplified representation of protein conformations for rapid simulation of protein folding. J. Mol. Biol. 104, 59-107.

Litchfield, J. T., and Wilcoxon, F. (1949). A simplified method of evaluating dose-effect experiments. J. pharmacol. Exp. Ther. 96, 99-113.

Martinez-Carrion, M., Sator, V., and Raftery, M. A. (1975). The molecular weight of an acetylcholine receptor isolated from Torpedo californica. Biochem. Biophys. Res. Commun. 65, 129.

Mulac-Jericevic, B., and Atassi, M. Z. (1986). Segment a182-198 of Torpedo californica acetylcholine receptor contains a second toxin-binding region and binds anti-receptor antibodies. FEBS Lett. 199, 68-74.

Neumann, D., Barchan, D., Safran, A., Gershoni, J. M., and Fuchs, S. (1986). Mapping of the α-Bungarotoxin binding site within the α-subunit of the acetylcholine receptor. Proc. Natl. Acad. Sci. 83, 3008-3011.

Noda, M., Takohashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Asai, M., Inayama, S., Miyata, T., and Numa, S. (1982). Primary structure of α-subunit precursor of Torpedo californica acetylcholine receptor deduced from cDNA sequence. Nature 299, 793-797.

Noda, M., Takashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, F., Asai, M., Inayama, S., Miyata, T., and Numa, S. (1983). Primary structure of beta and delta subunit precursors of Torpedo californica acetylcholine receptor from cDNA sequences. Nature 301, 251-255.

Nomoto, H., Takahashi, N., Nagaki, Y., Endo, S., Arata, Y., and Hayashi. K. (1986). Carbohydrate structures of acetylcholine receptor from Torpedo californica and distribution of oligosaccharides among subunits. Eur. J. Biochem. 157, 233-242.

Oblas, B., Singer, R. H., and Boyd, N. D. (1986). Location of a polypeptide sequence within the α-subunit of the acetylcholine receptor containing the cholinergic binding site. Mol. Pharmacol. 29, 649-656.

Raftery, M. A., Hunkapillar, M. W., Strader, C. D., and Hood, L. E. (1980). Acetylcholine receptor: Complex of homologous subunits. Science 208, 1454-1457.

Ratnam, M., Nguyen, D. L., Rivier, J., Sargent, P. B., and Lindstrom, J. (1986). Transmembrane topography of nicotinic acetylcholine receptor: Immunochemical tests contradict theoretical predictions based on hydrophobicity profiles. Biochemistry 25, 2633-2643.

Tsernoglou, D., and Petsko, G. A. (1976). The crystal structure of a post-synaptic neurotoxin from sea snake at 2.2 A resolution. FEBS Lett. 68, 1.

Tu, A. T., and Hong, B. (1971). Purification and chemical studies of a toxin from the venom of Lapemis hardwickii (Hardwick's sea snake). J. Biol. Chem. 246, 2772.

Yang, C. C., Chang, C. C., and Lion, I. F. (1974). Studies on the status of arginine residues in cobrotoxin. Biochim. Biophys. Acta 365, 1-14.

Zubay, G. L. (1983). Biochemistry. Addison-Wesley Publishing Co., Reading, Mass., 269-174.

DISTRIBUTION LIST

5 copies	Commander US Army Medical Research Institute of Infectious Diseases ATTN: SGRD-UIZ-M Fort Detrick, Frederick, MD 21701-5011
1 copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, MD 21701-5012
12 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100